

deposited, without restriction as to availability, in the permanent culture collection of the American Type Culture Collection at 12301 Parklawn Drive, Rockville, Maryland 20852, and has been assigned the accession number ATCC 31,267.

Cont.
a.

The above microorganism is illustrative of a strain of Streptomyces avermitilis which can be employed in the production of the C-076 compounds. However, such description also embraces mutants of the above described microorganism. For example, those C-076 producing mutants which are obtained by natural selection or those produced by mutating agents including X-ray irradiation, ultraviolet irradiation, nitrogen mustard or like treatments are also included within the ambit of this invention.

One example of such an organism is a strain of Streptomyces avermitilis MA 4848 which was isolated after irradiation with ultraviolet light of Streptomyces avermitilis MA 4680. A lyophilized tube and a frozen vial of this culture has been deposited in the permanent culture collection of the American Type Culture Collection, and they have been assigned the accession numbers 31272 and 31271 respectively. Slightly higher fermentation yields of C-076 have been obtained using this frozen stock as inoculum.

On page 25, following line 3, insert the following:

PREPARATION 1

P A 250 ml. baffled Erlenmeyer flask containing 50 ml. of the following medium:

Lactose	2.0%
Distiller's solubles	1.5%
Autolyzed yeast, Ardamine pH	0.5%
pH - before sterilization	7.0

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B is inoculated with the contents of one frozen vial of Streptomyces avermitilis MA 4848 and incubated on a rotary shaker at 28°C for 24 hours at 150 RPM.

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20 10 Ml. of the above fermentation media is employed to inoculate 500 ml. of the same medium as above in a 2 liter baffled Erlenmeyer flask. The fermentation media is incubated at 150 RPM on a rotary shaker at 28°C for 24 hours.

All of the foregoing media is employed to inoculate 467 liters of the following media in a 756 liter stainless steel fermentor:

Lactose	2.0%
Distiller's solubles	1.5%
Autolyzed yeast, Ardamine pH	0.5%
Polyglycol 2000	0.32 ml./liter
pH - before sterilization	7.0

B 20 The fermentation media is incubated at 28°C for 40 hours with an air flow 10 cubic feet per minute and an agitation rate 130 RPM.

P 230 Liters of the above media is employed to inoculate 4,310 liters of the following medium in a 5,670 liter stainless steel fermentor:

Dextrose	4.5%
Peptonized milk	2.4%
Autolyzed yeast, Ardamine pH	0.25%
Polyglycol 2000	2.5 ml./liter
pH - before sterilization	7.0

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PS The fermentation continues for 144 hours at 26°C with an air flow rate of 54.3 cubic feet per minute and agitation of 120 RPM.

Cont. 92
P The fermentation media are filtered and the mycelial filter cake washed with about 550 liters of water, the filtrate and washings are discarded. The filter cake is agitated with about 1500 liters of acetone for about one hour and filtered. The filter cake is washed with a mixture of about 150 liters of acetone and 40 liters of deionized water affording about 2000 liters of extract.

The foregoing fermentation and extraction is repeated on the same scale affording a further 2000 liters of acetone extract which is combined with the first extract and evaporated to a volume of about 800 liters. The pH of the concentrate is adjusted to about 4.7 with concentrated hydrochloric acid and combined with about 800 liters of methylene chloride. The combined solvents are agitated for about 4 hours and separated. The aqueous layer is combined with an additional 800 liters of methylene chloride and agitated for about 4 hours. The layers are separated and each methylene chloride extract separately treated with about 10 kilograms of Super-Cel and filtered. Both extracts are evaporated to a combined volume of about 60 liters.

P *a* PREPARATION 2

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The 60 liter solution of C-076 in methylene chloride of the previous example is concentrated to dryness in vacuo and the residue is combined 3 times with 60 liter portions of methanol and evaporated to dryness to remove any residual methylene chloride. The final methanol concentrate volume is approximately 36 liters. The methanol solution is stored overnight and filtered. The filter cake is washed with 40 liters of fresh methanol and the methanol filtrates and washings are combined. The methanol solution is combined with 95 liters of ethylene glycol and 130 liters of heptane. The 2 layer solution is agitated for 5 minutes and the lower layer (ethylene glycol and methanol) is separated. The heptane solution is washed with a mixture of 20 liters of ethylene glycol and 6.3 liters methanol. After five minutes of agitation, the lower layer is separated and combined with the first ethylene glycol/methanol extract. An equal volume of water (approximately 150 liters) containing 79 g. of salt per liter is added to the ethylene glycol/methanol extracts. This solution is extracted with 150 liters of ethyl ether with agitation for 5 minutes. The ether layer is washed with 75 liters of water (1/2 volume) and agitated for 5 minutes and the layers separated. This procedure is repeated an additional 2 times (the final water wash contains 20 g. of salt per liter) affording a final ether layer volume of 110 liters. The ether layer is concentrated in vacuo, to a minimum volume, keeping the temperature less than 25°C. 40 Liters of methylene chloride is added to the residue and the solution is evaporated to dryness. This

178

procedure is repeated and the final residue concentrated
20 in vacuo at 50°C to dryness.

CL PREPARATION 3

P A 30 centimeter diameter column is prepared with a layer of 34 kilograms of activated alumina followed by a layer of 34 kilograms of activated carbon in a solution of methylene chloride. The residue from the previous example is dissolved in methylene chloride to a volume of 34 liters and applied to the column and eluted with 34 liters of methylene chloride. these fractions are discarded. A 3% solution of isopropanol and methylene chloride (20.8 liters of isopropanol and 660 liters of methylene chloride) is applied to the column and eluted in approximately 200 liter fractions. The combined isopropanol and methylene chloride fractions are evaporated in vacuo at a bath
20 temperature of about 60°C to a volume of about 20 liters.
L The bath temperature is reduced to about 45°C and the extract is evaporated to dryness in vacuo. The residue is dissolved in 10 parts methylene chloride, 10 parts hexane and one part methanol to a final volume of 15 liters. This solution is applied directly to the Sephadex LH-20 column of the next example.

PREPARATION 4

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P A 30 centimeter diameter column is prepared in methanol with 36 kilograms of Sephadex LH-20 (available from Pharmacia Fine Chemicals, 800 Centennial Avenue, Piscataway, New Jersey 08854) and washed with a solvent consisting of 10 parts methylene chloride, 10 parts hexane and one part methanol. One-fourth of the C-076 solution of Example 10 is applied to the column and the column eluted at a rate of 250 ml. per minute. Two 20 liter forecuts are collected and discarded followed by 20 two liter rich cuts (identified as fractions 1-20), followed by a single 20 liter tail cut, which is discarded. Fractions 1-8 are found to contain the C-076 A compounds and fractions 9-20 are found to contain the C-076 B compounds.

*N*PREPARATION 5

P The process of Preparation 4 is repeated on the same scale three more times and all of the fractions containing the C-076 B components (fractions 9-20) are combined and evaporated to dryness, affording 818 g. of crude mixed C-076 B components. The sample is found to contain 55% C-076 B1 and 39% of C-076 B2. 680.5 G. of this sample is dissolved in 2 liters of methylene chloride and placed in a 22 liter three neck round bottom flask followed by the addition of 13.6 liters of methanol. The methylene chloride is removed by distillation. 13.6 Liters of ethylene glycol is added as the methanol is being distilled under reduced pressure. The rate of distillation is maintained such that the temperature of the solution

20 did not go below 65°C. When the addition of the ethylene glycol is complete, the solution is allowed to cool at 20 5°C for sixteen hours. The crystals are filtered and washed with 1 liter of cold ethylene glycol. The crystals are then redissolved in 2 liters of methylene chloride the solution placed in a 22 liter three necked round bottom flask. The procedure described above is repeated twice. The first time 12.5 liters each of methanol and ethylene glycol is employed and the second time 13.6 liters each of methanol and ethylene glycol is employed. The final crystals are washed with 1 liter of cold ethylene glycol and 1 liter of water. The crystals are dissolved in 4 liters of water and dried by filtering through sodium sulfate. The benzene solution is concentrated to a volume of 2 liters and lyophilized affording 241.2gm. of a white powder consisting of 98% C-076 B₁ and 1% of C-076 B₂.

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The mother liquors (22 liters) from the first two crystallizations above are combined and diluted with 22 liters of water. The aqueous solution is extracted with 60 liters of toluene and again with 15 liters of toluene. The toluene extract is then washed with 48 liters of water. The organic phase is filtered through Super-Cel to remove any residual water and evaporated affording 336 gm. of solid material consisting of 79% C-076 B₂ and 16% C-076 B₁ compounds.

P *u* PREPARATION 6

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In the four Sephadex LH-20 columns of the procedure of Preparation 4, fractions 1-8 contain the C-076 A compounds and are combined. By HPLC analysis the mixture is found to contain 252 g. of C-076 A2a, 16 g. of A2b, 94 g. of Ala and 24 g. of Alb. The material is dissolved in a solvent system consisting of hexane: toluene:methanol in the proportion of 6:1:1 and applied to the Sephadex LH-20 column of the same dimensions as the one used in Preparation 4 in the above solvent. Fractions are collected at the rate of 250 ml. per minute and a 20 liter forecut is collected and discarded. Further elution affords 2 additional 20 liter forecuts which are also discarded and 50 four liter rich cuts which contain C-076 A compounds. Fractions 3-8 are found to contain predominately C-076 A1 components *N* (40.2 g. Ala and 6.7 g. Alb), and fractions 29-36 are found to contain C-076 A2 compounds (117.2 g. A2a and 7.35 g. of A2b). Fractions 9-28 contain a mixture of C-076 A1 and A2 compounds.

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P *u* PREPARATION 7

A sample of 150 g. of C-076 B1 from Preparation 5 is dissolved in 3 liters of a solvent mixture of hexane: toluene:methanol in the ratio of 3:1:1. The solution is passed through a column of Sephadex LH-20 (of the same dimensions as the one used in Preparation 4) in the above solvent taking fractions at the rate of 250 ml. per minutes. After two 20 liter portions of the solvent mixture are collected and discarded, forecut of 10 liters is taken and discarded. Then 30 richcuts of 2 liters each

are taken. Fractions 1-13 and 25-30 are discarded. Fractions 14-16 are combined and contain 80 g. of predominately C-076 Bla. Fractions 22-24 are combined and contain 6.7 g. of predominately C-076 Blb. Fractions 17-21 contain a mixture of C-076 Bla and Blb.

Cont. 92
Fractions 17-21 above are combined and concentrated and passed through a Sephadex LH-20 column with the same solvent system as above. Three 20 liter forecuts are taken and discarded. Richcuts are then taken as follows: 5 cuts of 2 liters each (fractions 1-5); 20 cuts of 1 liter each (fractions 6-25); and 10 cuts of 2 liters each (fractions 26-35). Fractions 1-15 are discarded; fractions 16-21 contain 13.5 g. of C-076 Bla and 0.4 g. of C-076 Blb; fractions 22-26 contain 44 g. of C-076 Bla and 0.13 g. of C-076 Blb; fractions 27-30 contain 10.2 g. of C-076 Bla and 0.8 g. of C-076 Blb.

u PREPARATION 8

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20 *P* A mixture of all 8 C-076 components are chromatographed on a high pressure liquid chromatography column 4mm. X 30 cm. packed with 10 micron μ Bondapak C₁₈ silica gel (available from Waters Associates Inc., Maple Street, Milford, Massachusetts 01757) eluting with 85:15 (v/v) methanol:water at a constant 40°C. At a flow rate of 1.2 ml. per minute all eight compounds are separated and the elution volumes, which under the foregoing constant conditions are characteristic of the individual compounds are as follows:

	<u>Elution Volume (Ve) Ml</u>
C-076 B ₂ b	5.90
C-076 B ₂ a	6.52
C-076 A ₂ b	7.12
C-076 A ₂ a	7.88
C-076 B ₁ b	8.36
C-076 B ₁ a	9.60
C-076 A ₁ b	10.24
C-076 A ₁ a	11.88

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The separation of C-076 "b" components from the respective "a" components is accomplished using techniques such as high pressure liquid chromatography. An absolute methanol solution of 30 microliters of a mixture of C-076 Ala and Alb, estimated to contain 33 30 micrograms of C-076 Alb is placed on a 3x250 mm. high pressure liquid chromatography column containing Spherisorb 5 micron ODS (available from Spectra Physics) as packing. The column is eluted with 85:15 methanol³ water at a rate of 0.15 ml./min. The elution of the products are followed by observing the ultraviolet absorption of the eluent and collecting the individual components at the outlet of the UV monitor. 30 Micrograms of C-076 Alb is recovered in this manner.
